The Antibacterial and Immunomodulatory Effect of Zinc-Oxide Nanoparticles on *Pseudomonas aeruginosa* Infected Fresh-Water Fish

Eman Eldemerdash [a], Waleed Khalil[b], Nada Fayez[b*], and Abdel-Fattah Mohamed Abdel-Fattah [b]

[a] *Free Veterinarian.*

[b] *Pharmacology Department, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, 41522, Egypt.*

[*] *Corresponding author*

Email address: nada_fayez@vet.suez.edu.eg Phone number: +20 01026829925

Abstract:

In the recent decades, Zinc oxide nanoparticles (ZnONPs) are already used in several fields either for commercial or industrial purposes. In this study, 120 freshwater fish *(Oreochromis niloticus)* were divided into 4 different groups, each in triplicate groups (n=10), and used to assess the pharmacological activity of ZnONPs as an anti-bacterial and immunomodulatory agent. Initially, the antibacterial activity was detected *in-vitro* through the disk diffusion method. The results revealed a potent antibacterial activity, especially at 80 mg/kg of ZnONPs. Then, Pathogenicity test with *pseudomonas aeruginosa* was done. The clinical signs following the infection were also detected and the mortality rate was calculated. Administration of ZnONPs (80mg/kg) showed potent ability to protect the fish from mortality induced by I/P injection of *P. aeruginosa.* Finally, blood samples were collected twice, on the 20th and 40th days, to detect the effect of ZnONPs on some immune parameters including granulocyte, monocytes, lymphocytes and white blood cells (WBCs) count. The oral administration of both ZnONPs and zinc acetate resulted in a significant increase in lymphocyte, monocyte and WBCs count from the 20th to 40th days in infected tilapia. In contrast, the results of granulocyte count revealed that the counts decreased from 20th to 40th days in groups exposed to 80mg/kg ZnO NPs. In conclusion, ZnO-NPs showed significant antibacterial activity against *P. aeruginosa*-infected tilapia fish either *in-vitro* or *in-vivo* as well as elevated the immunity of the fish against *P. aeruginosa* infection.

Keywords: Antibacterial; Immunity; Nile Tilapia; *Oreochromis niloticus*; *Pseudomonas aeruginosa*.

1. Introduction

Although aquaculture has significantly contributed to food security by meeting the enormous need for animal protein, in recent decades the aquaculture industry has been very concerned about infections in fish that result in disease outbreaks and can cause significant economic harm due to morbidity and mortality. The massive expansion of fish farming in Egypt has highlighted a number of fish husbandry challenges, including fish diseases and their management which are frequently connected to stocking density that allows the widespread of harmful germs and are frequently a major factor in such major events **(Leung & Bates, 2013).** The bacterial infection with Aeromonas are one of the most predominant bacterial infections in fish species reared either in tropical habitats or in freshwater aquaculture, where they produce bacterial bleeding in cultured fish **(Semwal *et al.*, 2023)**

Oreochromis niloticus (O. niloticus) which is a worldwide distributed fish species and Egypt in particular, with high economic importance for fisheries and aquaculture, is also the most commercially important freshwater-farmed fish species in Egypt. but unfortunately, not far from other animals, fish usually get attacked by various types of diseases either due to pathogenic or non-pathogenic causes. Pathogenic Diseases are prime agents causing lethality in fish, especially when they are still in younger ages, stressed, overpopulated and polluting water resources. Among these pathogenic diseases is *Pseudomonas aeruginosa (P.aeruginosa)* which is classified as a Gram-negative bacterium that infects fish, particularly when they face unsuitable conditions leading to diseases mainly of ulcer type including, but not only, hemorrhagic septicemia and ulcerative syndrome present in soil and aquaculture environment **(S. Yaseen *et al.*, 2020).**

Although this bacterium is a normal inhabitant inside the fish, it becomes highly pathogenic and opportunistic under unsuitable conditions like mal-nutrition and or over-crowding resulting in many severe diseases such as splenomegaly, gill necrosis, abdominal distension, hemorrhagic septicemia. Furthermore, the increasing resistance of this bacterium to antibiotics makes the infection more difficult to be treated efficiently **(Algammal *et al.*, 2020)**.

The best methods for controlling infectious diseases of fish include the improvement of many management practices, the starting with a stock which can genetically resist infections, the attention to the dietary supplements and the movement between stocks, the avoidance of non-specific immunostimulant drugs , vaccines and other non-specific treatments **(Kumar *et al.*, 2015).** Currently, one of the riskiest actions is the frequent use of antibiotics in aquaculture and

fisheries especially those are already used as a human medication. Specialists, in turn gave some valuable advises to replace antibiotics use with other approaches to prevent infections. Such approaches include immunostimulants, growth enhancers, probiotics or vaccination programs **(Austin, 2017).**

A nanoparticle (NP) is commonly defined as a structure with a diameter ranged from 0.1 nm to 100 nm (1/1,000,000 mm). It is well documented that nanotechnology has many potential advantages particularly, in several sectors of industry, and goods such as cosmetics, paint, and automobiles and pharmaceutical industries **(Bhattacharyya *et al.,* 2016)** The testing of nanoparticles (iron, silver & selenium) on fish development and performance has revealed that nutraceutical delivery at the nanoscale can enhance fish growth **(Handy *et al.,* 2012)**. Here, it can be said that the manipulation of fish health depends on the use of nanotechnology, the nano-delivery of pharmaceuticals in diets and the use of nano-sensors for pathogen detection in aquaculture systems. Finally, for the controlling of medications as vaccines delivery and the assurance of getting a highly protected farmed fish especially against pathogens, nanotechnology should be widely used **(Nasr-Eldahan *et al.,* 2021).**

Zinc oxide nanoparticle (ZnO-NP) is an inorganic metal known as nontoxic with a good taste with growth-enhancing properties **(Faiz *et al.,* 2015)**. This nanoparticle fulfills all the following characteristics nontoxic, does not react with feed, has a good taste or tasteless and has not a bad smell. It is the third top worldwide distributed nano-metal after nano-silicon (nano-SiO2) and nano-titanium (nano-TIO2) **(Swain *et al.,* 2016).** It is identified as a Generally Recognized as Safe (GRAS) item by the Food and Drug Administration (FDA) **(FDA, 2016**)**.** So, it can be used safely as a medicine, and an antimicrobial agent in food packaging. Accordingly, we designed a work-oriented towards using nanomaterial such as zinc oxide nanoparticles for controlling this pathogenic disease.

2. Materials and methods

2. a. Materials:

The microbial strain (*Pseudomonas euroginosa*) was kindly supplied by Fish Health and Management Department, Central Laboratory for Aquaculture Research, Abbassa, Sharkia, Egypt. It was used in a concentration of 3x106 CFU/ml. Culture Media Roswell Park Memorial Institute 1640 medium (RPMI 1640) with L-glutamine and Nutrient Agar were purchased from Sigma-Aldrich, USA.

2. b. Experimental design:

The present study was performed in the Central Lab for Aquaculture

Research (CLAR), Al-Abassa, El-Sharkia governorate, Egypt.

A total number of 120 fish were reared in 12 full glass aquaria with dimensions of 70×60×50 cm, Fish under experiment were randomly divided into four different groups, each one, in 3 replicates (n=10). The first group (G1) was a control not infected and fed on standard fish diets /kg standard fish diet). The second group (G2) was infected with pseudomonas bacteria and then fed with a diet of 80mg nano-zinc oxide/kg. While the third group (G3) was infected with pseudomonas bacteria then treated with 80mg zinc acetate/kg standard fish diets). The fourth group (G4) was infected with pseudomonas bacteria then fed on standard fish diets. Fish was twice daily for 40 days at a daily rate of 3% of body weight. The immune parameters were measured at the experiment after (20 days) and at the end of the experiment throughout the period of the investigation (40 days).

2. c. Preparation of zinc oxide nanoparticles:

Zinc oxide nanoparticles was synthesized by reacting zinc acetate with a base in an alcohol solution. The reaction involved dissolving 3.942 g of zinc acetate and 1.44 g of NaOH in 1L of absolute ethanol and refluxing the mixture at 60˚ C for 3 hours. The reaction resulted in the conversion of zinc acetate to zinc oxide due to the reaction between the acetate group and the base. The synthesized ZnO was dispersed in an alcohol solution and was transparent and stable for at least 2 weeks.

After the previous reaction, the Zinc particles were dispersed in ethanol and then purified by mixing with DI water. The particles were separated from the dispersion supernatant through repeated centrifugation at 7000 rpm for 5 minutes. At the end, zinc particles were distributed again in DI water to obtain a water dispersion. The size of the ZnO particles was found to be between 5-20 nm based on TEM analysis according to **Wang *et al.* (2018)**

2. d. <u>Antibacterial sensitivity test</u>

According to **Essawi *et al.* (2020)** the susceptibility of the isolates to zinc acetate and nano zinc oxide was detected using a disc diffusion method, the susceptibility of the bacteria was determined according to the size of the inhibition zone, the method of preparation as follows:

1- Colonies from 24 hours' vintage subculture have been inoculated into about five ml of muller Hinton broth and kept for 4-5 hours until the turbidity became clear, and the bacterial suspension turned into adjusted to a density equivalent to that of MacFarland. Standard tube NO.5. 2- nutrient agar plate was prepared and dried earlier than inoculation and with the aid of using a sterile Pasteur pipette 0.1ml of bacterial suspension was spread onto a plate.

2- The plates have been stripped in different directions and the plate dried for as much as half an hour.

3- A sterile filter paper disc (9 mm diameter) was impregnated with 10 L of sterile zinc particles dissolved in diluted HCL and zinc acetate dissolved in distilled water at various concentrations (20,40,80) mg and placed into the inoculated agar surface. and with sterile pointed forceps gentle pressure over the disc to ensure complete contact of the disc to the medium then the plate turned into aerobically incubated at 37ºC for 24hours.

4- The degree of sensitivity measured by the diameter of the visible area of inhibition of growth around the disc.

2. e. Pathogenicity test

A pathogenicity test was done at the beginning of the feeding period. Challenge was applied by the technique called bath challenge in which a culture of pathogenic strain of *P. aeruginosa* was inoculated in 500 ml of Tryptic soya broth for 24 hrs. at 25ºC. The challenge process was applied in 3 groups (G2, G3 and G4) through IP route. Each fish received a 0.2 mL of a bacterial suspension ($3x10^7$ CFU/ml). Fish were transferred to their original aquaria incubation period of 24-72 hours and observed for 7 days post-challenge for any clinical abnormalities and mortalities. The mortality rate was recorded in both infected and control fish groups and the relative percentage of survival (RPS) was calculated as reported by **Amend (1981)**

Relative percent of survival (RPS): -

$$\textbf{RPS= 1-}\frac{\%\textit{of infected mortality}}{\%\textit{ of control mortality}} \times \textbf{100}$$

2. f. Hematology (Blood sampling):-

Fish blood samples were collected after 20 days and 40 days from the blood vessels using a disposable tuberculin syringe. Where, detected white blood cell (WBC), monocyte, granulocyte and lymphocyte count were measured based on **Spada *et al.* (2019)**

2. f. 1. Monocytes, granulocytes and lymphocytes count: -

It was calculated using the Mann-Whitney test and was based on differences in mononuclear count (MNC) determined by the peripheral smear method and flow cytometry plots was used for the counting of CD34. To estimate the MNC threshold in peripheral blood and PBSC product for optimal mobilization and harvest, the receiver operating characteristic curve was used, according to **Krishnan *et al.* (2021)**

2. f. 2. Separation of white blood cells: -

White blood cells separation was carried out according to the method described by **Anderson *et al.* (1979)** In brief, diluted blood mixture was slowly layered onto Ficoll-Hypaque medium (3 ml) in a 16 x 97 mm clear plastic tube and centrifuged at 500 xg for 30 min at room temperature in a wing-fixed bucket centrifuge. The lower band at the interface,

comprising white blood cells were harvested with a plastic pipette and washed twice with calcium and magnesium-free PBS. Cells were counted in a hemocytometer and then resuspended in one ml of RBMI medium followed by the addition of one ml of fetal bovine serum (FBS). The white blood cells were adjusted to $2x10^6$ cells/ml in PBS.

2. h. Statistical analyses:

One-way ANOVA was used to analyze the results. Then, Duncan's new multiple-range test was used to test for mean differences at the 5% probability level. SPSS V.10 (SPSS, Richmond, USA) was used for all statistical analyses. as described by **Bewick *et al.* (2004).**

3. Results

3. a. Antibacterial sensitivity test to nano-zinc oxide

Overall, the results of both nano-zinc, zinc acetate, Amikacin and Ampicillin antibacterial sensitivity test against *p.aeruginosa* showed positive results represented in diameters of inhibitory zones in all except ampicillin was resist. Meanwhile, The strongest antibacterial action was very clear in amikacin followed by nano-zinc and zin acetate, the diameter of the inhibitory zone of (80, 40 and 20) % nano-Zno were (5,3 and 1) mm respectively and the diameter of the inhibitory zone of 80,40 and 20 % zinc acetate are (3,1 and 0.5) mm, respectively. On the other hand, the diameter of inhibitory zone of amikacin was (1.1 cm). Also, ampicillin showed resistance. As shown in **fig. (1,2 and 3).**

3. b. Clinical signs after I.P. injection of p. aeroginosa in experimental fish:

As shown in **Fig (4).** Fish injected intraperitoneally with *P. aeruginosa* suffered from petechial hemorrhage, skin darkness, focal necrosis, separated scales, body edema and exophthalmia and fin erosion **(fig. 4a)**, mild tail hemorrhage and necrosis **(fig. 4b)**, exophthalmia, corneal opacity, tail hemorrhage and necrosis **(fig. 4c);** darkness of the skin, detached scales, exophthalmia and all fin erosion **(fig. 4d).**

3. c. The effect of nano-zinc oxide on mortality of fish challenged with Pseudomonas bacteria

Administration of which received ZnONPs (80mg/kg) showed potent ability to protect the fish from mortality induced by I/P injection of P. aeruginosa represented in stopping of the mortality just after treatment application G2 followed by G3 which received (80mg/kg) zinc acetate showed a stop of mortality 2 days after treatment. On the other hand, G4 (control positive) which did not receive treatment showed continuous mortality for continuous 4 days as shown in, **Table (1).**

3. d. The effect of nano-zinc oxide on immunological parameters:

3. d. 1. Effect of nano-zinc oxide on lymphocyte count-

Pseudomonas infection in Nile tilapia significantly ($P<0.05$) elevated lymphocyte for 20 days but

non-significant elevated for a long time 40 days. Oral administration of both zinc acetate and ZnONPs non significantly reduced lymphocytes in infected tilapia compared with infected non-treated group, **Table (2).**

3. d. 2. Effect of nano-zinc oxide on monocyte count: -

Pseudomonas infection in Nile tilapia significantly (P<0.05) elevated monocytes for a long time (up to 40 days) compared with a normal non-infected non-treated group (G1). Oral administration of both ZnONPs and zinc acetate significantly reduced monocytes in infected tilapia compared with an infected non-treated group (G4). Reduction was observed for 20 only not for 40 days. On the other hand, both zinc acetate and ZnONPs significantly (P<0.05) reduced monocytes in diseased tilapia compared with the non-treated group (G1) and this reduction observed after 40 days, **Table (3)**.

3. d. 3. Effect of nano-zinc oxide on granulocyte: -

Pseudomonas infection significantly (P<0.05) reduced granulocytes compared with the normal non-infected non-treated group (G1). Meanwhile, 40 days after *Pseudomonas* infection, granulocytes were significantly (P<0.05) lower than normal values observed in the non-infected group (G1). Compared with the infected non-treated group (G4), both zinc acetate and ZnONPs supplementations significantly (P<0.05) elevated granulocytes in infected tilapia; these significant elevations continued and became more prominent after 40 days of zinc supplementation, **Table (4)**.

3. d. 2. Effect of nano-zinc oxide on WBCs count: -

The Pseudomonas infection in Nile tilapia significantly (P<0.05) elevated WBCS for 20 days) but not for 40 days. Oral administration of zinc acetate significantly reduced WBCS in infected tilapia compared with the infected non-treated group (G4) meanwhile there was not any significant change after 40 days, as shown in **Table (5).**

Table (1): *showing the effect of nano-zinc oxide on mortality of fish challenged with pseudomonas bacteria*

Groups	No. of treated fish	No. of mortality after treatment	Mortality % after treatment	No. of survival after treatment	Survival % after treatment
G1	**30**	**0**	**0%**	**30**	**100%**
G2	**27**	**0**	**0%**	**27**	**100%**
G3	**23**	**2**	**8.69%**	**21**	**91.31%**
G4	**26**	**8**	**30 .76%**	**18**	**69.24%**

(G1) fed on basal diet free from zinc (control negative), (G2) infected Fish on basal diet provided with 80mg/kg ZnONPs, (G3) infected Fish on basal diet provided with 80mg*/Kg* zinc acetate, (G4) infected Fish fed basal diet free from zinc (control positive).

Table (2): *Changes lymphocyte count of O. niloticus treated with nano-zinc oxide and zinc acetate*

Groups	Lymphocyte %	
	20 days	40 days
G1	83 ± 0.45^{b}	83 ± 0.45^{b}
G2	86.33 ± 0.93^{a}	88.33 ± 1.37^{a}
G3	84 ± 0.45^{ab}	86 ± 0.45^{ab}
G4	86 ± 0.45^{a}	85.33 ± 0.68^{ab}

A total number of 120 fish divided into 4 groups: G1 fed on basal diet free from zinc (control negative), G2 infected Fish on basal diet provided with 80mg/kg ZnONPs, G3 infected Fish on basal diet provided with 80mg/Kg zinc acetate, G4 infected Fish fed basal diet free from zinc (control positive). Columns with different superscript letters are

The superscript letters within the columns represent statistical difference at $P<0.05$.

Table (3): *Changes in monocyte of O. niloticus treated with nano-zinc oxide and zinc acetate:* **-**

Groups	Monocytes %	
	20 days	40 days
G1	1.50 ± 0.22^{b}	1.50 ± 0.22^{b}
G2	2.50 ± 0.22^{b}	3.50 ± 0.39^{a}
G3	1.83 ± 0.47^{b}	3.00 ± 0.22^{a}
G4	5.33 ± 0.52^{a}	3.50 ± 0.39^{a}

A total number of 120 fish divided into 4 groups: G1 fed on basal diet free from zinc (control negative), G2 infected Fish on basal diet provided with 80mg/kg ZnONPs, G3 infected Fish on basal diet provided with 80mg/Kg zinc acetate, G4 infected Fish fed basal diet free from zinc (control positive).

The superscript letters within the columns represent statistical difference at $P<0.05$.

Table (4): *Changes in granulocyte of O. niloticus treated with nano-zinc oxide and zinc acetate:* **-**

Groups	Granulocytes%	
	20 days	40 days
G1	15.17 ± 0.85^{a}	15.17 ± 0.65^{a}
G2	15.83 ± 1.05^{a}	14.50 ± 0.81^{a}
G3	13.17 ± 0.56^{a}	11.00 ± 0.77^{b}
G4	9.50 ± 0.67^{b}	8.00 ± 0.45^{c}

A total number of 120 fish divided into 4 groups: G1 fed on basal diet free from zinc (control negative), G2 infected Fish on basal diet provided with 80mg/kg ZnONPs, G3 infected Fish on basal diet provided with 80mg/Kg zinc acetate, G4 infected Fish fed basal diet free from zinc (control positive). The superscript letters within the columns represent statistical difference at P<0.05.

Table (5): *Changes WBCs count of O. niloticus treated with nano-zinc oxide and zinc acetate: -*

Groups	WBCs (10^3/µl)	
	20 days	40 days
G1	22.33± 0.68^{b}	23.67 ± 0.93^{a}
G2	24.00 ± 0.89^{ab}	26.67 ± 1.13^{a}
G3	22.67 ± 0.93^{b}	24.00 ± 1.18^{a}
G4	26.67 ± 1.13^{a}	23.33 ± 0.93^{a}

A total number of 120 fish divided into 4 groups: G1 fed on basal diet free from zinc (control negative), G2 infected Fish on basal diet provided with 80mg/kg nano-zinc oxide, G3 infected Fish on basal diet provided with 80mg/Kg zinc acetate, G4 infected Fish fed basal diet free from zinc (control positive). The superscript letters within the columns represent statistical difference at P<0.05.

Fig. (1) inhibitory zone of three concentration (80, 40 and 20) % of nano-zinc oxide against *p. aeruginosa*.

Fig. (2) inhibitory zone of three concentrations of zinc acetate (80, 40 and 20)% against *p. aeruginosa*.

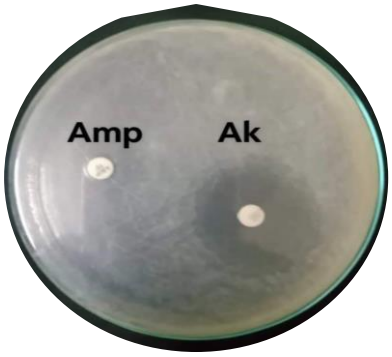

Fig. (3). sensitivity of *pseudomonas* against amikacin (inhibitory zone) and resistance against Ampicillin.

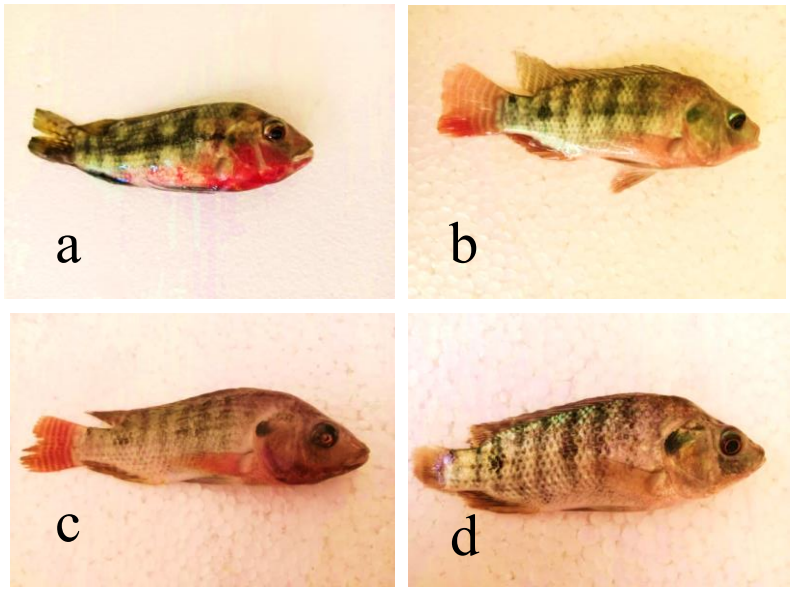

Fig. (4) Showing charactaristic lesions of fish injected intraperitoneal with *P. aeruginosa.* (a) Showing petechial hemorrhage, darkness of the skin, focal necrosis, detached scales, abdominal ascites, exophthalmia and fin erosion. **(b)** showing mild tail hemorrhage and necrosis. **(c)** showing exophthalmia, corneal opacity, tail hemorrhage and necrosis. **(d)** showing darkness of the skin, detached scales, exophthalmia and all fin erosion

4. Discussion

Fish reared in intensive culture are adversely impacted and frequently susceptible to various microbial infections that have been manipulated with antimicrobial agents, including antibiotics. Campaigns to restrict antimicrobial use and look for alternatives have arisen as a result of the growth of the problem of microbial

resistance to antibiotics and the harm it brings to human and animal health. **(Robertson *et al.,* 2000)**. The current investing of nanoparticles in aquaculture for preventing and treating various diseases is undoubtedly a progress and will certainly solve or even minimize the massive and frequent use of antibiotics. The present work aimed to investigate the effect of ZnONPs on bactericidal activity and immunological parameters of Nile tilapia.

In support of the immune stimulatory effects shown for ZnONPs in this study is the protection against disease induced by pathogenic *Pseudomonas aeruginosa* injected I/P in *O. niloticus* where the relative level of protection reached 100% just after ZnONPs administration in doses of 80mg/kg diet (G2). Zinc oxide nanoparticles have potential bactericidal effects on gram-negative and gram-positive bacteria. Such bactericidal activity could be attributed to efficient transport of ZnO nanoparticles through the plasma membrane resulting in cell death resulting from the higher surface area for interaction with the bacterial surface **(Auffan *et al.,* 2009).** Since the discovery that FtsZ is a protein that coordinates all aspects of cell division and cell wall remodeling. **Tripathy & Sahu (2019)** and **Mendes *et al.* (2022)** examined the likelihood of zinc particles to interfere with the membrane and cell division components of *B. subtilis* (*amy*: pspac-ftsZ-gfpmut1) expressing FtsZ-GFP. The findings proved that ZnO NPs did not inhibit the divisional Z-ring assembly. Though, they mentioned that 15 min post contact with zinc particles, around 70% of the cells faced damage in the cytoplasmic membrane. Moreover, the production of zinc ions and synthesis of free radicals (electrostatic forces) were discussed as a potential mechanism of ZnO bactericidal action. In addition, **Han *et al.* (2021)** demonstrated that the protein plays as a critical target of many bactercial agents and it mainly works during cell replication to organize the cytoplasmic ring inside the pathogen**.** Another theory explains that the bactericidal action of Zno as the reduction of FtsZ in the replication cascade.

Since that hematological evaluations are becoming a routine practice for determining health status in fish, our results showed a significant increase in WBC, lymphocyte, and monocyte count were noticed after 40 days of ZnONPs supplementation in G2 and organic zinc G3 in comparison with G4. The explanation for these results comes from the fact that zinc particles modulates the response of the immunity and this modulation is controlled by many other parameters and transporters. Once the previous methods is interrupted, the concentrations of zinc particles is decreased which in turn alter the cells survival, integrity, replication and differentiation of different organs and systems, especially those of the immune system **(Bonaventura *et al.,* 2015).**

5. Conclusion

Zinc oxide nanoparticles protected tilapia against mortalities induced by P. aeruginosa infection. Such protection attributed to the proved antibacterial activity of ZnO NPs shown in-vitro as well to the enhanced immunity manifested by significant increase in lymphocyte, monocyte and WBC count along the experimental period

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الملخص العربي

في الدراسة الحالية قمنا بدراسة التأثير المعملي للتركيزات المختلفه من جزيئات النانو زنك اكسيد علي بكتيريا السيدوموناس ومن ثم على القياسات المناعية ومقاومة الامراض للبطى النيلى .أجريت الدراسة على أسماك البلطي النيلي (*Oreochromis niloticus*) بوزن 116 ± 1.7 جم/سمكة وطول 2 ± 10سم تم جمعها من أحواض المعمل المركزي لبحوث الثروة السمكية بالعباسة أبوحماد شرقية (تم الاحتفاظ بعدد 120 سمكة في 12 حوض زجاجي) ، حيث تم استخدام هذه الأحواض لعمل مجموعات الاسماك (3 أحواض / مجموعة) تم تزويد الاحواض بمياه خالية من الكلور. كان متوسط درجة الحرارة 25 درجة مئوية ± 1 درجة مئوية وتم ضبط الاكسجين للتهوية المستمرة باستخدام ضواغط ضخ الهواء الكهربائية. تم تأقلم الاسماك تحت الظروف المعملية في خزانات داخلية لمدة أسبوعين للدراسة التجريبية. قسمت الاسماك المختبرة إلى أربع مجموعات ، كل مجموعة ، على ثلاث تكرارت ، كل مجموعة تحتوي على 10 أسماك المجموعه الاولي (لم يتم اجراء أي معاملات معمليه عليها واطعامها علف سمكي قياسي تم حقن المجموعة الثانيه ببكتيريا السيدوموناس ومعالجتها بجزيئات النانوزنك المجموعة. الثالثه حقنت ببكتيريا السيدوموناس وتمت معالجتها ب 80مج/كج زنك اسيتات . بينما عوملت المجموعة الرابعه بحقنها بكتيريا السيدوموناس وعلف سمكي قياسي. تم تغذية الاسماك ثلاث مرات في اليوم لمدة 40 ا بمعدل يومي ً 3 ٪ من وزن الجسم. تم تغيير مياه الحوض يوميا وتم سحب عينات دم مرتين خلال التجربه بينهما 20يوم التصميم التجريبي على النحو التالي :المجموعة الاولي تتغذى على النظام الغذائي الاساسي بدون اضافات المجموعة الثانيه تتغذى على النظام الغذائي الاساسي المكمل بنسبة 80مج من النانو زنك اكسيد/كجم علف .تم تغذية المجموعة الثالثه على نظام غذائي أساسي مكمل بنسبة 80من اسيتات الزنك /كجم علف. المجموعة الرابعة تتغذى على النظام الغذائي الاساسي بدون اضافات

1- زاد العد الكلي للخاليا اللمفاوية معنويا في المجموعات التي تناولت جزيئات الزنك 80% كجم علف سمكي.

2- ازداد العدد الكلي للمينوسيد (monocyte) . بعد 20يوم في المجموعة التي تلقت 80مج/كج علف نانو زنك.

3- زادWBC عدد كرات الدم البيضاء معنويا.

- مما تقدم تشير نتائجنا بوضوح شديد إلى التأثير التحفيزى المناعي للنانو زنك اكسيد في البلطي النيلي.
- كما توصي الدراسة باستخدام النانو زنك في النظام الغذائي الاسماك لما لها من آثار مفيدة على أداء الاسماك